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14. ABSTRACT: AKT/PKB represents potentially a promising molecular target for the design of new anticancer therapies for the treatment of human breast cancer and other types of cancer. In this DOD Idea Award grant, using computational approach, we have discovered a class of potent small-molecule inhibitor of AKT/PKB (API-59). API-59 selectively inhibits AKT activity and has little effect on other upstream protein kinases or MAP kinases. API-59 is highly potent and effective in inhibition of cell growth and induces apoptosis in human breast cancer cell lines with high levels of AKT and has selectivity over cancer cells with low levels of AKT. Importantly, API-59 has little toxicity to normal cells. Our present study provides a solid proof-of-the-concept that targeting AKT or AKT pathway in human breast cancer is a promising strategy for the design and development of a class of anticancer therapy, and API-59 represents a promising lead compound for further evaluation and optimization..

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INTRODUCTION

AKT, or protein kinase B (PKB) is a serine/threonine kinase discovered in 1991, that was shown to play an important role in growth factor mediated cell survival. Inappropriate activation of AKT/PKB has been associated not only with cancer development but also confers resistance of cancer cells to current therapeutic agents. It has also been shown that activation of AKT promotes cancer metastasis. AKT inhibits apoptosis in tumor cells by multiple mechanisms, including inhibiting pro-apoptosis protein Bax conformational change, phosphorylation of several other components of the apoptotic machinery, including BAD and caspases 9. AKT cooperates with anti-apoptotic Bcl-2 member, Bcl-X_L to promote cancer cell survival. Taken together, these studies have provided strong evidence that inhibition of AKT or AKT-mediated signal transduction pathway is an attractive strategy for the design and development of new anticancer drugs for the treatment of human breast and other types of cancer.

This DOD Idea grant aims at identification and characterization of small-molecule inhibitors of AKT. Below, we summarize what we have accomplished on this grant.

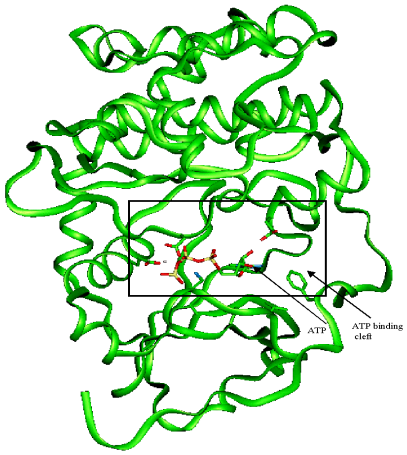
Of note, this award was initially made in 2001 when Dr. Dajun Yang (the initial principal investigator) in Georgetown University. Dr. Dajun Yang moved his research laboratory to the University of Michigan in February 2002. Dr. Yang requested a transfer of the award from Georgetown University to the University of Michigan, which was approved in June 2003. In 2005, Dr. Yang has taken a leave of absence from his academic position from the University of Michigan to work full time for a start-up company. The University of Michigan requested a transfer of the principal investigator to Dr. Shaomeng Wang, which was approved in Nov, 2005 by the DOD Breast Cancer Program.

BODY OF REPRT

1. Computational homology modeling of AKT structure

When the time we started this project, the experimental structure of AKT/PKB kinase domain was not determined. We have first therefore modeled the three-dimensional structure of AKT3 using computational homology modeling approach. Sequence analysis using BLAST identified that the kinase domain of AKT had a high sequence homology with several Ser/Thr kinases (40% sequence identity and 60% sequence similarity) to AKT kinase domain. Using homology modeling program, MODELLER, developed by Dr. Sali's group, we modeled the 3D structure of AKT kinase domain. In addition, we have performed extensive structural refinement using computational molecular dynamics (MD) simulations in water using the CHARMM program. Ribbon representation of the refined structural model for AKT/PKB (isoform 3) is shown in Figure 1.

Figure 1. Ribbon representation of modeled structure of AKT/PKB (isoform 3) through computational homology modeling. The ATP binding site is highlighted.

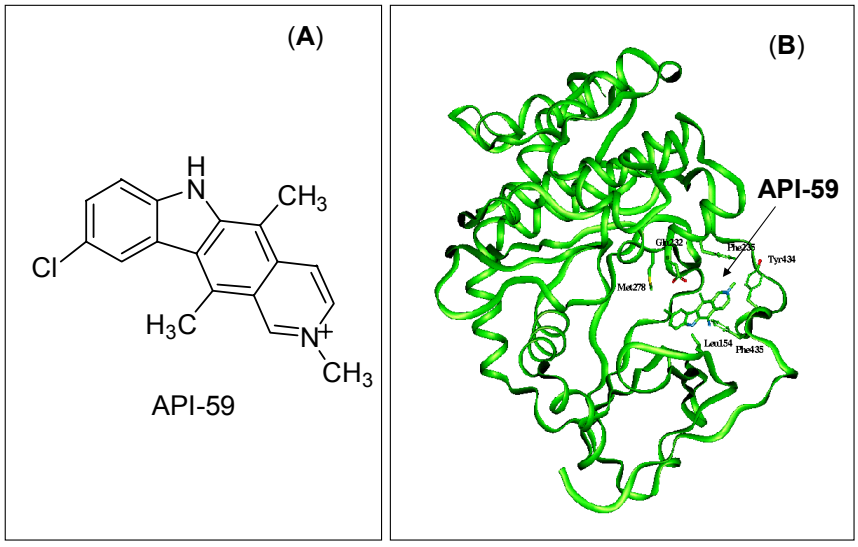


2. Structure-based database searching

Over the years, we have built extensive three-dimensional structural databases of druglike, small organic compounds. Using the structural model of AKT/PKB kinase domain, we have searched the database of over 650,000 small organic compounds using the DOCK program, developed by Professor Kuntz at University of California at San Francisco. We selected the DOCK program for its fast speed and reasonable accuracy in predicting the binding models of small-molecules in complex with their protein targets. Based upon the predicted docking scores, we have selected the top 200 compounds for experimental screening.

1.3. Computational docking studies of confirmed lead compounds

Figure 2. (**A**). Chemical structure of a promising lead compound (API-59) identified from database searching. (**B**). Predicted binding model of API-59 in complex with AKT by computational docking.



We have performed extensive docking studies to predict the binding models for a number of lead AKT inhibitors we have discovered. These included the most promising lead compound API-59. The chemical structure of API-59 and the predicted binding model of API-59 in complex with AKT are shown in **Figure 2**.

3. Comprehensive characterization of the expression status of AKT and PTEN in human breast cancer and other types of cancer cell lines

To facilitate our studies of AKT inhibitors on their activity, selectivity and mechanism of action, we have undertaken the task to characterized the expression status of AKT, phospho-specific AKT, and PTEN in NCI 60-cancer cell lines by Western blot analysis using antibodies against AKT, phospho-Ser473-AKT, and PTEN.

There is no difference among NCI-60 cancer cells regard to their AKT expression (data not shown), but the expression of phospho-Ser473-AKT and PTEN are quite different in these 60 cell lines. Among them, SNB19 and SF-539 glioma cancer cells express the highest level of phospho-Ser473-AKT, whereas they do not express PTEN or express undetectable level of PTEN; HS578T breast cancer cells expresses high level of phospho-Ser473-AKT, but it expresses a relatively high level of PTEN; MCF-7 breast cancer cells do not express phospho-Ser473-AKT, but it expresses the highest level of PTEN. MDA-MB-453 breast cancer cell line, one of the model cell lines used in this work also expresses high level of AKT and PTEN (data not shown). These results suggest that the expression of phospho-Ser473-AKT, which also stands for phospho-Thr308-AKT, is unique in each of the cancer cell lines and can be used as a target for drug development.

4. Characterization and confirmation of potential AKT inhibitors with AKT kinase assay, ELISA, and Fluorescence Polarization-based IMAP^{TM} kinase assay

We utilized a sensitive and quantitative *in vitro* AKT kinase assay (New England Biolab) to identify cell lines with highest level of AKT kinase activity. The principle behind this assay is to use an antibody against AKT to selectively immunoprecipitate AKT from cell lysates. The resulting immunoprecipitated enzyme is then incubated with GSK-3 fusion protein in the

presence of ATP and kinase buffer. In this reaction mixture, GSK-3 is phosphorylated and subsequently detected by Western blot with a Phospho-GSK-3 (Ser21/9) antibody. Selective analysis of GSK-3 phosphorylation at Ser21/9 gives rise to an improved specificity with high sensitivity and near zero background. This assay allowed us to confirm the potential inhibitors under either cell-free condition (adding the inhibitors directly in the kinase reaction mixture) or in the intact cells (treating cells in culture medium with inhibitors first).

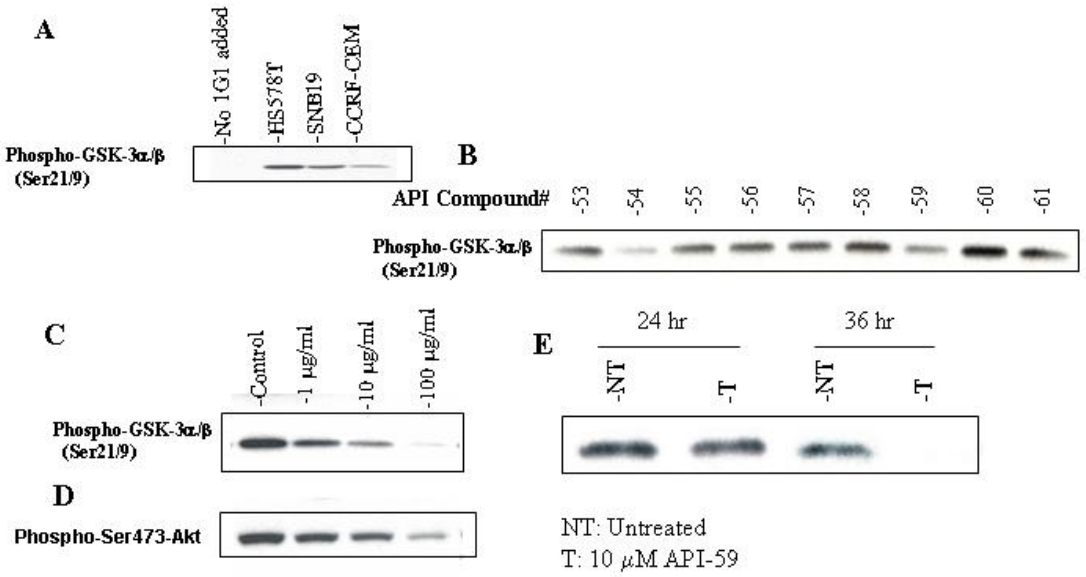


Fig. 3 A: HS578T shows the highest AKT activity. 100 μg lysates of HS578T, SNB19, and CCRF-CEM cells were subjected to AKT kinase assay. **B**. AKT inhibitor (API) screening with AKT kinase assay 100 μg lysates of HS578T cells were immunoprocepitated with 1G1 AKT antibody, the immunoprecipitated AKT was used to conduct AKT kinase assay in the presence of 10 μg/ml of different AKT inhibitor candidates. **C**: Dose-Dependent AKT inhibition by API-59. 2×10^5 HS578T cells were treated with desired concentrations of API-59 for 30 min, cell lyseates were harvested and subjected to AKT kinase assay. **D:** API-59 inhibits phospho-Ser473 AKT. Same lysates were subjected to Western blot analysis with phospho-Ser473 AKT antibody. **E**: Time-course of the inhibitory effect of API-59. 2 × 105 HS578T breast cancer cells were treated with 10 μM API-59 for various times, cell lyseates were subjected to AKT kinase assay.

Using this method, we immunoprecipitated AKT (monoclonal antibody 1G1, which recognizes AKT1, AKT2, and AKT3) from breast cell line Hs578T which has highest level of activated AKT kinase (**Fig. 3A**) to screen a total of 69 candidate small molecule inhibitors from top-200 compounds selected from our computational screening, as shown in F**igure 3B**. We found 5 compounds displayed a specific kinase inhibition for AKT with an IC_{50} from 1 to 10

μg/ml. In order to test the cell permeability of those compounds, we then carried out the cell based AKT kinase inhibition assay by treating HS578T cells in culture with those 5 lead compounds. One of which **API-59,** has an IC_{50} value <10 μg/ml. The dose and time-dependent inhibition of **API-59** is shown in **Fig. 3C and 3E**. Inhibition of AKT kinase activity also led to reduction of phosphorylation of AKT (**Fig. 3C)**, when the same cell lysates was investigated with phospho-Ser473 AKT antibody by western blot analysis.

To verify API-59 does inhibit phospho-Ser473 AKT, we performed an Enzyme-Linked ImmunoSorbent Assay (ELISA)-based assay to investigate whether API-59 inhibits the activity of phospho-Ser473 AKT in MDA-MB-453 human breast cells. As shown in **Figure 4**, treated with various concentrations of API-59 for 24 hrs, the activity of phospho-Ser473 AKT was significantly decreased with 20 μM of API-59 treatment, and was completely blocked with 40 μM of API-59 treatment.

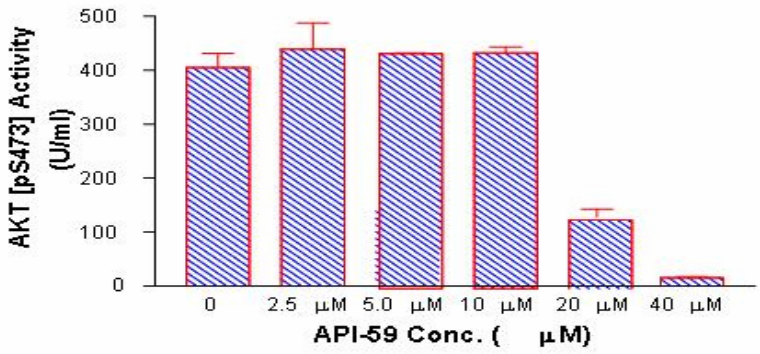


Fig. 4 1× 106 MDA-MB-453 cells were treated with API-59 for 24 hrs, cells lysates were subjected to an ELISA-basedAKT [pS473] activity assay.

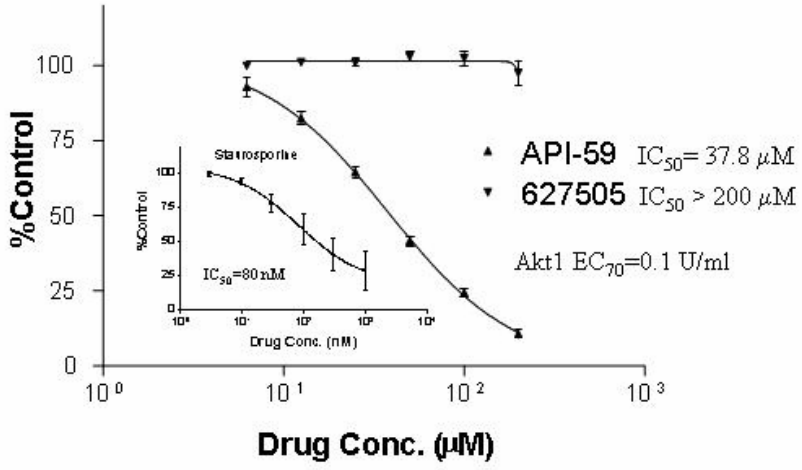


Fig. 5 Fluorescence polarization-based IMAP TM AKT1 assay. Different Concentrations of API-59 or NSC627505 were subjected to IMAP TM kinase assay against AKT1.

The IMAP AKT Assay kit (Molecular Device, Sunnyvale, CA) was designed to evaluate kinase activity of AKT. It employed the IMAP technology for non-antibody fluorescence polarization (FP) detection of phosphorylation. The IMAP technology is based on the high affinity binding of phosphate by immobilized metal (MIII) coordination complexes on nanoparticles. This IMAP "binding reagent"complexes with phosphate groups on phosphopeptides generated in a kinase reaction. Such binding causes a change in the rate of the molecular motion of the peptide, and results in an increase in the fluorescence polarization values observed for the fluorescein label attached at the end of the peptide. Specifically, first to get the EC70 value of AKT by preparing an enzyme dilution curve with adding 5 μl of Complete Reaction Buffer, serial diluted AKT enzyme, fluorescein-labeled substrate (final concentration 0.1 μM), and ATP (final concentration 5 μM) in one well of a 384-well plate (Corning, NY), reaction at room temperature for 1 hr, adding 60 μl IMAP binding regent, reaction at room temperature for 30 min, then reading the plate with Tecan Ultra microplate reader set at

fluoresecence polarization mode. Second to get IC_{50} values of candidate AKT inhibitors by setting up the same assay with serial dilution of inhibitors against AKT with the activity of EC_{70}.Purified AKT1 enzyme is now commercially available (Upstate). With this method, we directly detect the inhibitory effect of API-59 in regard to its inhibition against AKT1-mediated SGK substrate phosphorylation. As seen in **Figure 5**, positive control staurosporine inhibits AKT1-mediated SGK substrate phosphorylation with IC_{50} 80 nM; while API-59 shows an inhibitory effect against AKT1 with IC_{50} 37.8 μM. An inactive control (NSC 627505) does not inhibit AKT1.

Taken together, we demonstrated that API-59 inhibits the activity of AKT in a dose and time-dependent fashion using multiple, complementary assays.

5. Determination of the selectivity of API-59 for inhibition of AKT over other kinases

To demonstrate that our identified lead the lead compound API-59 selectively inhibits the AKT kinase but not other kinases, we used the same cell lysates from human breast cancer HS578T cells treated with API-59 to probe with antibodies against phosphor-tyrosin kinases, phospho-specific MAP kinase, and PI-3 kinase. As shown in **Fig. 6**, API-59 does not inhibit these target proteins. These results suggest that **API-59** inhibits AKT activity in cancer cells with high-levels of AKT activity, API-59 selectively inhibits the AKT kinase activity and has no or little effect on the upstream protein kinases of AKT or MAP kinase.

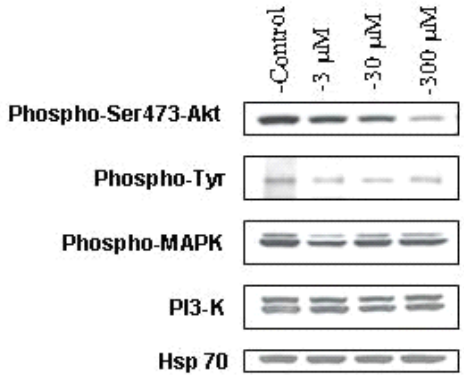


Fig. 6 API-59 inhibits only AKT, not inhibit protein tyrosine kinases, PI3K, or phospho-p42/p44 MAP kinase. 2×10^5 HS578T cells were treated with desired concentrations of API-59 for 30 min, cell lysates were harvested and subjected to AKT kinase assay and Western blot analysis. Hsp70 reprobing is showing equal loading of the total protein samples.

5. The inhibition of AKT activity by API-59 is ATP-dependent

AKT/PKB is a serine/threonine kinase and ATP is required for its function. To evaluate whether ATP is also required for the inhibition of AKT activity by API-59, we performed an AKT kinase assay with different concentrations of GSK-3 fusion protein (substrtate) and ATP in the presence of API-59, using AKT immunoprecipitated from HS578T cells. As can be seen in **Figure 7**, at 0.5 or 1 μg of GSK-3 fusion protein and in the absence of API-59, ATP increased AKT kinase activity in a dose-dependent manner, as indicated by the phosphor-GSK-3 band. With 1.2 and 6 μM of API-59, ATP was still able to increase AKT kinase activity. However, in the presence of 30 μM API-59, ATP failed to increase AKT kinase activity. This finding suggested that API-59 may be an ATP competitive inhibitor, consistent with its predicted mode of action by our computational docking studies.

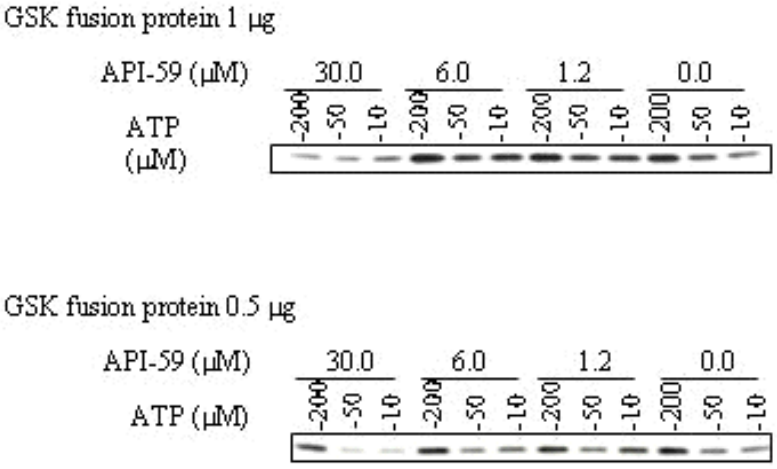


Fig. 7 The AKT inhibitory effect of API-59 can be reversed by ATP. 250 μg of HS578T cell lysates were used to immunoprecipated AKT, then subjected to kinase assay with desired concentrations of ATP and GSK-3 fusion protein in the presence of different concentrations of API-59.

6. API-59 inhibits ligand-induced activation of AKT

In tumor cells, AKT can be activated by a variety of growth factors and cytokines. We thus tested the ability of API-59 in blocking heregulin (HRG) induced AKT activation in human breast cancer MCF-7 cells. As shown in **Figure 8A**, treatment with 30 nM of Heregulin for 30 min readily induced activation of AKT in human breast cancer MCF-7 cells, which usually expresses low or undetectable level of AKT, treatment of these cells again with 30 μg/ml of API-59 significantly reduced such activation. Furthermore, as predicted, blocking of AKT activation also resulted in subsequent reduction of phosphorylation of pro-apoptotic protein BAD, which is down-stream substrate of AKT (**figure 8B**). If this effect in cells by API-59 is due to its ability to block the AKT activation, this effect would not affect proteins in a different pathway. To test this hypothesis, we then tested the ability of API-59 in inducing the activation of MAP kinase in the same system. As shown in **figure 8B**, heregulin readily induced activation of MAP kinase, which

is consistent with reports in the literature and our previous data [64]. However, API-59 had no effect on the MAP kinase activation induced by ligand heregulin. These results illustrate that API-59 selectively inhibits ligand-induced activation of AKT.

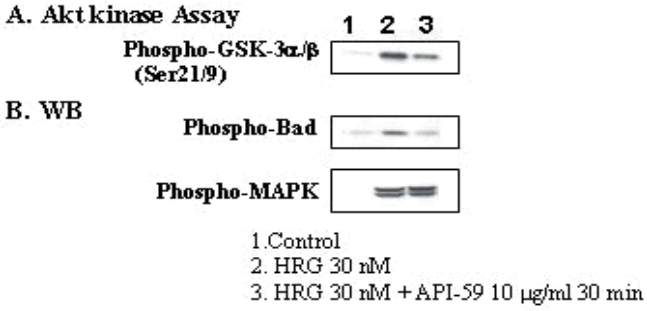


Fig. 8 API-59 inhibits heregulin-induced activation of Akt. **A:** Heregulin induces activation of AKT in MCF-7 cells, but this activation is inhibited by API-59. 3×10^5 MCF-7 Cells were treated as indicated in the picture, cell lystates were harvested and subjected to AKT kinase assay. **B**: API-59 inhibits heregulin-induced up-regulation of phospho-Bad, but does not afftect the activation of phospho-MAP kinase. Same lysates were subjected to Western blot analysis with phospho-Bad and phospho-p42/p44 MAP kinase specific antibodies, respectively

7. API-59 selectively inhibits the growth of breast cancer cells with high AKT activity

It was predicted that inhibition of AKT activity in human cancer cells with high levels of AKT activity will result inhibition of cell growth. We have therefore performed experiments to determine the ability of API-59 in a panel of human cancer cell lines with different AKT activity. We have also tested API-59 for its selectivity in human mammary epithelial cells (HMEC).

Cells were treated with a series of doses of API-59 with regular culture medium supplemented with 10% FBS for 5-7 days and then cell viability was determined by Cell Counting Kit-8 (**Figure 9**). The rationale of this kit is similar to that of the MTT assay, but with a novel tetrazolium salt that produces a water-soluble formazan dye upon bioreduction. As seen in **Figure 9**, API-59 is potent and effective in the MDA-MB-468 (MDA-468) and MDA-MB-

453 (MDA-453) human breast cancer cell lines with high levels of AKT activity. API-59 has an IC_{50} value of 80 nM in the MDA-MB-468 cell line and 200 nM in the MDA-MB-453 cell line, respectively. In comparison, API-59 is ineffective in inhibition of cell growth in the MCF-7 cell line, which has low levels of AKT, and has an IC_{50} value >5 μM. Very importantly, APK-59 has very little toxicity to human mammary epithelial cells (HMEC). Taken together, our data demonstrate that API-59 selectively inhibits the growth of human breast cancer cells with high levels of AKT activity and has little toxicity to normal cells.

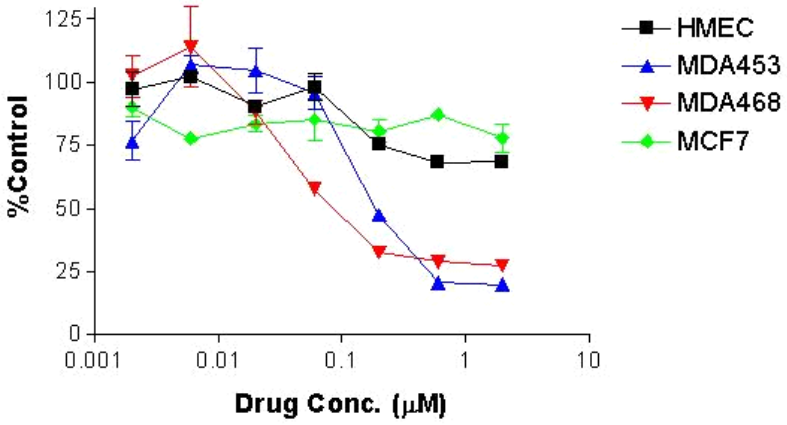


Fig. 9 API-59 inhibits the growth of cancer cells with high Akt activity. 5 × 105 MDA-MB-453, MDA-MB-468, and MCF-7 breast cancer cells were treated with different concentrations of API-59 for 5 days, then cell viability was tested with Cell-Counting Kit-8.

8. API-59 selectively induces apoptosis of cancer cells with high AKT activity

We next investigated if API-59 is capable of inducing human breast cancer cells with high levels of AKT activity to undergo apoptosis using TUNEL staining, which specifically stains the breakage of DNA.

As can be seen in **Figure 10**, treatment by 2 μM of API-59 for 72 hours failed to induce apoptosis in NIH-3T3 cells, which have very low levels of AKT activity. In contrast, the same

treatment induced 55% MDA-MB-453 cells, which have high levels of AKT activity, to undergo apoptosis. In addition, API-59 caused DNA fragmentation in almost MDA-MB-453 cells (**Figure 10D**).

Taken together, API-59 is highly effective and selective in induction of cell death in human breast cancer MDA-MB-453 cells with high levels of AKT activity and has little effect in the NIH-3T3 cells with low levels of AKT.

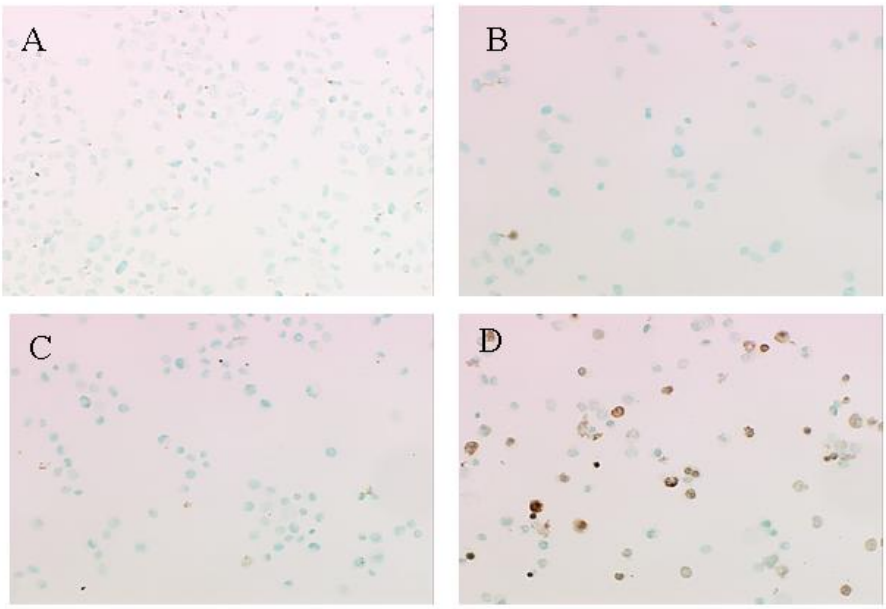


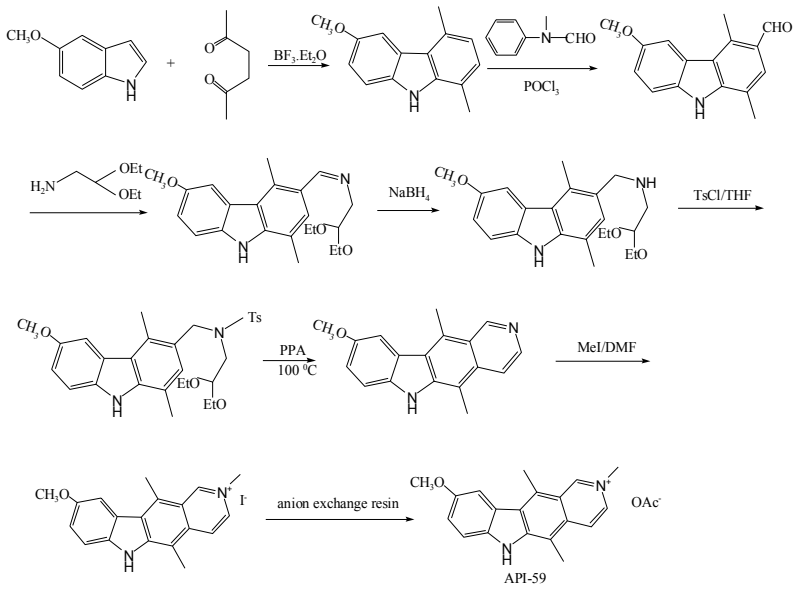
Fig. 10 API-59 induces apoptosis of cancer cells with high AKT activity. 1×10^6 MDA-MB-453 cells (high AKT activity) and 5×10^5 NIH3T3 cells were treated with 2 μM API-59 for 72 hrs, cells were then subjected to TUNEL staining. **A**. Untreated NIH3T3 cells. **B**. Treated NIH3T3 cells. **C**. Untreated MDA-MB-453 cells. **D**. Treated MDA-MB-453 cells.

9. Chemical synthesis of API-59 and chemical synthesis of its analogues

Our in vitro studies have provided strong evidence that API-59 may represent a promising lead compound to inhibit the activity of AKT in human breast cancer cells. To further assess its therapeutic potential, *in vivo* studies in animal models of human breast cancer are needed. To this end, we have developed a synthetic procedure for the synthesis of gram quantity

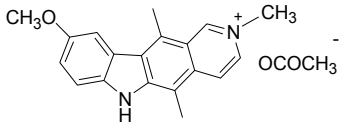
of API-59. The synthetic procedure is shown in **Figure 11**. Using this procedure, we have synthesized 500 mg of API-59. Synthesis of additional 2-3 grams of API-59 is ongoing.

Figure 11. Synthesis of API-59 and its analogues.

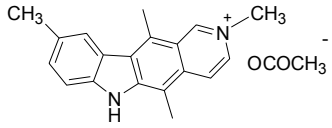


Using the same synthetic procedure, we have synthesized additional analogues of API-59, who chemical structures are shown in **Figure 12**. Our initial evaluation of some of these new analogues such as API-59-CJ-Cl is as potent as API-59 and some of these analogues have an improved druglike properties such as solubility such as API-59-CJ-OMe. We plan to carry out detailed characterization of these new analogues in human breast cancer models for the activity, selectivity and mechanism of action.

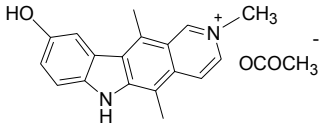
Figure 12. Chemical structures of new analogues of API-59.



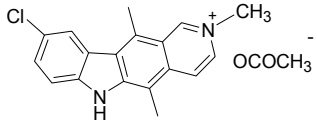
API-59-CJ-OMe



API-59-CJ-Me



API-59-CJ-OH



API-59-CJ-Cl

Key Research Accomplishments

In this DOD Idea Award grant, using computational approach, we have discovered a class of potent small-molecule inhibitor of AKT/PKB (API-59). API-59 selectively inhibits AKT activity and has little effect on other upstream protein kinases or MAP kinases. API-59 is highly potent and effective in inhibition of cell growth and induces apoptosis in human breast cancer cell lines with high levels of AKT and has selectivity over cancer cells with low levels of AKT. Importantly, API-59 has little toxicity to normal cells. Our present study provides a solid proof-of-the-concept that targeting AKT or AKT pathway in human breast cancer is a promising strategy for the design and development of a class of anticancer therapy, and API-59 represents a promising lead compound for further evaluation and optimization.

REPORTABLE OUTCOMES:

Manuscripts:

1. Discovery and characterization of API-59 as a potent and selective AKT/PKB inhibitor, Manchao Zhang, Ribo Guo, Jingsong Wang, Lei Shao, Istvan Envedy, Yeyu Cao, Yan Ling, Jane Wu, Zhujun Yao, Shaomeng Wang*, and Dajun Yang*, manuscript completed and in final revision (to be submitted to Cancer Research).
2. Design and synthesis of new analogues of API-59 as a new class of AKT/PKB inhibitors. Jianyong Chen, Dajun Yang, Shaomeng Wang, manuscript in preparation (to be submitted to Journal of Medicinal Chemistry).

Meeting Abstracts

Specific blockade of AKT/PKB activity in cancer cells with a selective AKT/PKB inhibitor API-59, Authors: Zhang M, Guo R, Wu X, Liu H, Wang S, Yang D. FASEB Summer Research Conference- Protein Kinases and Protein Phosphorylation, July 19-24, 2003, Snomass, CO.

Collaborations:

In addition to our own investigations of API-59 in human breast cancer models, we have made API-59 and one of its analogues available to collaborators in the US and in Germany. These collaborations have resulted in three publications. These studies have extended our finding from human breast cancer models into other cancer models and shed new lights on the mechanism of action.

1. Jin X, Gossett DR, Wang S, Yang D, Cao Y, Chen J, Guo R, Reynolds RK, Lin J. Inhibition of AKT survival pathway by a small molecule inhibitor in human endometrial cancer cells. British Journal of Cancer, **2004** Nov 15;91(10):1808-12.
2. H.J. Tang, X. Jin X, Shaomeng Wang, D. Yang, Y. Cao, J. Chen, D. R. Gossett, J. Lin, A small molecule compound inhibits AKT pathway in ovarian cancer cell lines, Gynecol Oncol. **2006,** 00(2):308-17.
3. Mahmoud Toulany, Ulla Kasten-Pisula, Ingo Brammer, Shaomeng Wang, Jianyong Chen, Klaus Dittmann, Michael Baumann, Ekkehard Dikomey, and H. Peter Rodemann, Blockage of Epidermal Growth Factor Receptor-Phosphatidylinositol 3-Kinase-AKT Signaling Increases Radiosensitivity of K-RAS Mutated Human Tumor Cells In vitro by Affecting DNA Repair, Clinical Cancer Research, **2006** 12: 4119-4126.

CONCLUSIONS

AKT/PKB represents potentially a promising molecular target for the design of new anticancer therapies for the treatment of human breast cancer and other types of cancer. In this DOD Idea Award grant, using computational approach, we have discovered a class of potent small-molecule inhibitor of AKT/PKB (API-59). API-59 selectively inhibits AKT activity and has little effect on other upstream protein kinases or MAP kinases. API-59 is highly potent and effective in inhibition of cell growth and induces apoptosis in human breast cancer cell lines with high levels of AKT and has selectivity over cancer cells with low levels of AKT. Importantly, API-59 has little toxicity to normal cells. Our present study provides a solid proof-of-the-concept that targeting AKT or AKT pathway in human breast cancer is a promising strategy for the design and development of a class of anticancer therapy, and API-59 represents a promising lead compound for further evaluation and optimization.